Methods of Diagnosing, Prognosticating and Treating Matrix Metalloproteinase-1 Related Diseases via a Matrix Metalloproteinase-1 Single Nucleotide Polymorphism

INTRODUCTION

5   This invention was made in the course of research sponsored by the National Institutes of Health.   The U.S. Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

10   Matrix metalloproteinases (MMPs) are a family of at least 15 enzymes that degrade the extracellular matrix (ECM) (Borden, P. and Heller, R. Crit. Rev. Eukaryotic Gene Expr. 7: 159-178, 1997). These enzymes have essential roles in modeling and remodeling the ECM in normal physiology and

15 disease pathology. Several of these enzymes have the unique ability to degrade the interstitial collagenase (types I, II, and III), the body's most abundant proteins. MMP-1 is the most ubiquitously expressed interstitial collagenase, thereby assigning it a prominent role in collagen degradation.

20 Overexpression of MMP-1 is associated with several pathological conditions, including the irreversible degradation of cartilage, tendon, and bone in arthritis (Vincenti et al. Crit. Rev. Eukaryotic Gene Expr. 6:391-411, 1996) and the degradation of collagenase I and III in tumor

25 invasion and metastasis (Chambers, A.F. and Matrisian, L.M. J. Nat'l Cancer Inst. 89:1260-1270, 1997; Murray et al. Nat.

Med. 2:461-462, 1996). Patients with tumors that express MMP-1 have an overall poorer prognosis than patients with tumors that do not express this protein (Murray et al. Nat. Med. 2:461-462, 1996; Murray et al. J. Pathol. 185:256-261, 1998).

5   This overexpression of MMP-1 has been suggested to be due to the juxtaposition of transcription factor binding sites within the promoter of this gene and to the cooperativity among the factors that bind these sites (Buttice et al. Oncogene 13:2297-2306, 1996; Basuyaux et al. J. Biol. Chem. 272:26188-

10   26195, 1997; Gutman, A. and Waslyk, B. EMBO J. 9:2241-2246, 1990; Benbow, U. and Brinckerhoff, C.E. Matrix Biol. 15:519-526, 1997).

Most normal cells express modest, but detectable, levels of MMP-1 constitutively, and this expression increases

15   substantially in the presence of cytokines or growth factors (Vincenti et al. Crit. Rev. Eukaryotic Gene Expr. 6:391-411, 1996; Rutter et al. J. Cell Biochem. 66:322-336, 1997; Aho et al. Eur. J. Biochem. 247:503-510, 1997; Delany, A.M. and Brinckerhoff, C.E. J. Cell Biochem. 50:400-410, 1992).

20   However, A2058 melanoma cells constitutively express high levels of MMP-1 (Templeton et al. Cancer Res. 50:5431-5437, 1990), making them a useful model for studies on the transcriptional regulating of this gene and for comparative studies with normal cells.

25   A 4 kb region of the MMP-1 promoter DNA from a leukocyte genomic library was isolated and sequenced (Rutter et al. J. Cell Biochem. 66:322-336, 1997). DNA sequence analysis revealed that this clone contained only 1 G at position -1607 bp, resulting in the sequence 5'-AAGAT-3' (SEQ ID NO: 1)

30   (Rutter et al. J. Cell Biochem. 66:322-336, 1997). This sequence differs from that reported by others (Aho et al. Eur. J. Biochem. 247:503-510, 1997; Imai et al. Mol. Cell Biol. 14:7182-7194, 1994), wherein 2 Gs at that location which create the sequence 5'-AAGGAT-3' (SEQ ID NO: 2) are

35   described. The presence of 2 Gs at this site creates the

sequence 5'-GGA-3', which is a consensus sequence (Graves, J.B. Science 279:1000-1001, 1998) for a functional PEA3/EBS. This site has now been established to constitute a single nucleotide polymorphism (SNP). The full length DNA sequence
5 of MMP-1 with only 1 G at position -1607 is depicted in SEQ ID NO: 3.

Based upon the effect of this SNP on the transcriptional activity, protein/DNA binding activity, and frequency of this SNP in normal fibroblasts and in melanoma tumor cells it has
10 now been determined that detection of this SNP is useful in diagnosing and prognosticating cancer and other MMP-1 related diseases. It is also now believed that agents identified as inhibitors of binding of transcription factors to the Ets transcription factor binding site created by or resulting from
15 this SNP will be useful in treating MMP-1 related diseases.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method of diagnosing MMP-1 related diseases in a patient which comprises detecting in a patient MMP-1 containing an Ets
20 transcription   factor   binding   site   single   nucleotide polymorphism (MMP-1 EBS-SNP).

Another object of the present invention is to provide a method of prognosticating MMP-1 related diseases in a patient which comprises detecting in a patient suffering from
25 an MMP-1 related disease an MMP-1 EBS-SNP.

Another object of the present invention is to provide a kit for diagnosis and prognosis of an MMP-1 related disease in a patient via detection of MMP-1 EBS-SNP in a patient sample.
30 Another object of the present invention is to provide a method of identifying agents with potential therapeutic value in treating MMP-1 related diseases which comprises screening agents for the ability to inhibit the binding of a transcription factor to an MMP-1 EBS-SNP.

Yet another object of the present invention is to provide a method of treating MMP-1 related diseases in a patient which comprises administering to the patient an agent which inhibits binding of a transcription factor to an MMP-1
5 EBS-SNP.

DETAILED DESCRIPTION OF THE INVENTION

The abundance of single nucleotide polymorphisms (SNPs) in the genome make these genomic variations powerful tools for
10 identifying disease genes, particularly in loss of heterozygosity studies in tumors. A large effort is presently underway for identifying SNPs. Most research is focused on the coding regions of genes (Wang et al. Science 280:1077-1082, 1998).

15 However, an SNP located in the promoter of the interstitial collagen degrading enzyme, MMP-1, has now been identified. Because this variation is not located in a coding region, it does not alter the structure of the enzyme. However, the location of this SNP in the promoter region of
20 MMP-1 has now been demonstrated to have profound effects on the production/regulation of the enzyme. Further, an increase in the frequency of this SNP in tumor cell lines has now been demonstrated which is indicative of this SNP causing an increase in invasive behavior due to high levels of MMP-1
25 expression. MMP-1 is implicated in tumor cell invasion and metastasis due to its ability to cleave the interstitial collagenase types I and III at neutral pH. Therefore, a structural variation with the potential to influence the level of expression is important to understanding how this enzyme
30 modulates ECM metabolism and tumor cell invasion and metastasis. Further, detection of this SNP in a patient is useful in diagnosing and prognosticating MMP-1 related diseases.

The effects of this SNP, referred to herein as MMP-1
35 EBS-SNP, on the transcriptional activity and protein/DNA

binding activity, along with the frequency of this SNP in normal fibroblasts and in melanoma tumor cells were examined in detail.

5 Constitutive mRNA expression of endogenous MMP-1 gene in normal foreskin fibroblasts (HFS) and in the A2058 melanoma cells over a 24 hour period was determined. HFS expressed low levels of MMP-1 mRNA, while A2058 cells expressed higher levels. To determine whether the A2058 cells contained a difference in the endogenous MMP-1 promoter, polymerase chain

10 reaction (PCR) was used to amplify the promoter from these cells. This sequence was then compared with the previously isolated leukocyte clone described by Rutter et al. J. Cell Biochem. 66:322-336, 1997 and set forth in SEQ ID NO: 3 and with promoters from other sources (Aho et al. Eur. J. Biochem.

15 247:503-510, 1997; Imai et al. Mol. Cell Biol. 14:7182-7194, 1994). Several substitutions were observed, but were considered insignificant because they did not create or delete any known binding sites for transcription factors within the promoter. However, one major difference was detected: the

20 A2058 promoter DNA contained an additional G at position -1607 bp, which was flanked by a guanidine (5') and an adenine (3'), thus creating an Ets transcription factor binding site (EBS; Graves, B. Science 279:1000-1001, 1998).

To specifically test the role of the 1 G/2 G variation

25 in regulating transcription, two luciferase reporter constructs driven by a large (4.3 kb) fragment of the MMP-1 promoter were generated with the only difference being 1 G or 2 Gs at -1607 bp. This SNP is adjacent to an AP-1 site -1602 bp, which may also influence transcription. These clones were

30 transitely transfected in HFS and the effect on basal transcription was measured. A significant increase (ranging from 2-10-fold) in transcription with the 2 G promoter construct compared with the 1 G promoter construct was consistently observed in at least four separate donors of HFS.

35 Hirt's analysis of transfected DNA demonstrated that these

differences were not attributable to transfection efficiency. When these two constructs were transfected into the A2058 melanoma cells, a 29-fold increase in transcription of the 2 G construct over the 1 G construct was observed. Other

5 experiments showed similar increases ranging from 1- to 37-fold. These constructs were then tested in other tumor cell lines (MDA231 breast cancer cells, and two primary melanoma lines) to assess their transcriptional response. In these cells, the 2 G promoter construct augmented transcription at

10 least 4-fold over the 1 G construct. Thus, the increase in transcription is dependent on the presence of 2 Gs at -1607 bp, and it is seen in both normal and malignant cells.

The ability of an oligo probe containing either 1 G or 2 Gs at -1607 bp to bind to nuclear extracts from the A2058

15 cells was also assessed. A striking difference in binding intensity was seen, with the 2 G oligo binding more nuclear proteins compared with the 1 G oligo. Thus, the presence of the 2 Gs clearly augments this binding. Cross competition experiments were used to determine the specificity of binding

20 to each labeled oligo. Binding to the 1 G oligo was readily competed, suggesting that these DNA/protein interactions are weak. Competition studies with the 2 G oligo revealed that the "self" oligo only mildly competed the binding of the shared bands, although it did compete two bands, demonstrating

25 that these proteins bind preferentially and/or specifically to the 2 G oligo . Furthermore, the 1 G oligo did not compete well, but was effective in competing the shared bands. Finally, the proximal EBS-AP-1 competitor showed a pattern similar to that seen with the 1 G oligo. Taken together,

30 these observations indicate that the 2 G "self" oligo competes for the proteins able to bind specifically to the 2 gene sequence, and that the other bands represent proteins or protein complexes that are not 2 G-dependent (e.g. the AP-1 proteins). Importantly, these data also indicate that the

presence of the EBS in this region of MMP-1 creates an
environment where DNA/protein interactions strongly occur.

Because the SNP at -1607 bp is located adjacent to an
AP-1 site at -1602 bp, binding to the 1 G and 2 G oligos by
5 recombinant ETS-1 and c-JUN, proteins that are likely
candidates for binding to these sites, was investigated. The
recombinant proteins, by themselves, were not able to bind to
either oligo. The combination of both proteins, however,
bound to the 2 G oligo, but only faintly to the 1 G oligo,
10 suggesting that additional proteins were required for optimal
DNA binding. Indeed, incubation with nuclear extract from
A2058 cells resulted in distinctive binding patterns for each
probe. Complexes I, II, VI, and VII were present in both
panels, whereas complexes III, IV, and V were specific for the
15 2 G oligo. When ETS-1 and c-JUN were added together with
nuclear extract, binding to the 2 G oligo was more pronounced
compared with the 1 G oligo.

In the presence of recombinant c-JUN, complex I became
more apparent with both oligos, indicating that complex I may
20 represent AP-1 proteins binding to the DNA. Complex VII also
became more intense when c-JUN was added indicating that it,
too, contains AP-1 related proteins. When both recombinant
proteins were added, the binding pattern in the 1 G panel did
not differ from the reactions with c-JUN alone, supporting the
25 importance of 2 Gs in creating the EBS. When nuclear extracts
and recombinant ETS-1 were added to the 2 G oligo, a new
complex was formed (complex IV). Furthermore, when both
proteins and nuclear extract were added to the 2 G probe,
several complexes (I, III, IV, V, and VI) were diminished and
30 seemed to combine into a much stronger complex II, again
demonstrating the influence of the 2 Gs in creating EBS.

Antibodies to several members of the Ets family of
transcription factors (ETS-1/2, Erg-1/2, Elk-1 and ERM) were
used in "super/shift" reactions to identify the proteins
35 binding to this site. However, binding of these antibodies

was not detected due either to limitations of the antibodies, because the complexes did not allow for the antibody to have access to the epitope, and/or because these proteins did not bind. These data suggest that the oligos containing 2 Gs

5 represents a bona fide EBS that binds an Ets family member(s) in a complex with AP-1 protein members. While the data show that recombinant ETS-1 binds, it is believed that other Ets family members can also bind to this site.

Confirmation of this 1 G/2 G difference in the leukocyte

10 clone sequence and the A2058 melanoma sequence, to be a SNP and not a mutation, was performed. A radiolabeled PCR assay using primers that flank the variation to amplify a product of either 148 bp (1 G), 149 bp (2 G), or both (heterozygous) in genomic DNA was developed. One hundred control DNAs

15 derived from the CEPH pedigrees (http://www.cephb.fr/cephdb/) were then assayed to determine the frequency of this variation within a population. Only the parents in the pedigrees were used to avoid biasing the results through inheritance. In addition to the CEPH control DNAs, the frequency of this SNP

20 in several tumor cell lines, including the A2058 melanoma cells, was assessed. The occurrence of 2 G homozygotes in the CEPH controls was determined to be approximately 30%. In the tumor cells lines, it is 62.5% (P<0.0001).

Accordingly, based upon these experiments, it is

25 believed that the 1 G/2 G SNP influences the transcriptional responsiveness of the human MMP-1 promoter in cancer, where excessive production of MMP-1 is a major contributor to the stromal degradation involved in tumor invasion. As with other genes, expression of MMP-1 is meditated by multiprotein

30 complexes that bind to DNA in a sequence-specific manner, and these complexes often cooperate to achieve maximal activation. DNA elements containing a single EBS are often not sufficient for Ets induction, and require a nearby AP-1 site to which Fos and JUN proteins bind. Furthermore, the transcriptional

35 environment within the cells may influence the level of gene

expression, as evidenced by differences in the level of transcription of the 2 G allele in HFS versus melanoma cells. These differences may be due to various extracellular stimuli, such as growth factors and cytokines, as well as cell-type-

5 specific nuclear factors within the cell. Thus, the combination of cis-acting sequences in the MMP-1 promoter and specific trans-acting factors can dramatically increase transcription. This increase is believed to provide a molecular mechanism for enhanced ECM degradation not only in

10 cancer, but in other MMP-1 related diseases such as arthritis, cardiovascular disease and periodontitis.

For example, rheumatoid arthritis, a chronic disease that afflicts more than two million individuals in the United Stated and 1% of the population world-wide is characterized

15 by severe and irreversible degradation of cartilage, tendon and bone mediated by overexpression of MMPs. Inflammatory cytokines such as interleukin-1 and tumor necrosis factor-α activate synovial fibroblasts that line the joint and adjacent chondrocytes to produce these MMPs. Destruction of

20 interstitial collagen occurs mainly through two MMP family members, MMP-1 and MMP-13. MMP-1 and MMP-13 are expressed by synovial fibroblasts and articular chondrocytes. It is believed that, in similar fashion to patients suffering from cancer, overexpression of MMP-1 in patients suffering from

25 rheumatoid arthritis may result at least in part from this SNP.

Accordingly, detection of the MMP-1 EBS-SNP in a patient provides a useful means for diagnosing MMP-1 related diseases. Detection of MMP-1 EBS-SNP in a patient is indicative of the

30 patient suffering from a disease relating to overexpression of the MMP-1 enzyme. Detection of this SNP can be performed in accordance with well known techniques including, but not limited to, PCR as described herein.

Detection of MMP-1 EBS-SNP also provides a useful method

35 for prognosticating MMP-1 related diseases in a patient. For

example, it is well known that MMPs are key players in tumor
invasion and metastasis. Both processes require degradation
of the extracellular matrix, which is accomplished by
proteolytic enzymes that are secreted by either tumor cells,
5 themselves, and/or neighboring stromal cells. In fact, at
least for melanoma cells which produce MMP-1, the prognosis
of the disease is correlated with tumor thickness and depth
of invasion through dermal collagens (I and II), thereby
indicating that invasiveness through these collagens serves
10 as a valid prognostic marker. Accordingly, detection of MMP-1
EBS-SNP, which is indicative of enhanced ability to degrade
collagen types I, II and III, in tumor cells of a patient
serves as a useful prognostic marker in assessing the
invasiveness of a particular tumor. This prognostic marker
15 is thus useful in determining various treatment regimes
expected to be most successful in individual patients.

        Means for detecting MMP-1 EBS-SNP in a patient sample
for diagnosing and/or prognosticating MMP-1 related diseases
can be incorporated into a kit for easy use by a laboratory
20 technician. In one embodiment, the kit can comprise PCR
primers such as those described in Example 5 herein which
flank the MMP-1 EBS SNP. In this embodiment, the kit may also
comprise dGTP, dATP, dTTP, and dCTP; Taq DNA polymerase; and
α(32)P-dCTP. However, other means for detecting the MMP-1
25 EBS-SNP which can be incorporated into a kit will be obvious
to those of skill in the art upon this disclsoure. Kits of
the present invention may also comprise positive and negative
control samples.

        Further, inhibiting MMP-1 synthesis by targeting either
30 the MMP-1 EBS-SNP or proteins that bind to this SNP represents
a useful therapeutic approach to inhibit pathologic expression
of MMP-1, but not its normal physiological role. Potential
therapeutic agents for treatment of MMP-1 related diseases
including, but not limited to, cancer, rheumatoid arthritis,
35 cardiovascular disease and periodontitis, can be identified

by determining their ability to either bind to MMP-1 EBS-SNP,
to bind to proteins which bind to MMP-1 EBS-SNP or to inhibit
binding of MMP-1 EBS-SNP with other proteins. Agents
identified by this method can then be administered to patients
5 suffering from an MMP-1 related disease to alleviate the
symptoms resulting from overexpression of MMP-1.

       The following nonlimiting examples are provided to
further illustrate the present invention.

EXAMPLES

10 **Example 1: Cells and Plasmids**

       Primary human foreskin fibroblasts (HFF) were prepared
in accordance with procedures described by Rutter et al. J.
Cell Biochem. 66:322-336, 1997, and used during passages 4-8.
HFFs and A2058 cells were cultured in DMEM (Life technologies,
15 Inc.) with 10% fetal bovine serum (Sigma Chemical Co.),
penicillin (100 units/ml), and streptomycin (100 µg/ml).

       A 4.3-kb MMP-1 promoter DNA fragment containing only 1G
at -1607 bp was described by Rutter et al. J. Cell Biochem.
66:322-336, 1997. Primers were made to amplify the endogenous
20 promoter from the A2058 cells (-4008 bp to -3988 bp sense
primer: 5'-GTGGAAGCTTACACCTATAATCCCAACACTC-3' (SEQ ID NO: 4)
and -511 bp to -543 bp antisense primer: 5'-
CTGCCTGGTACCCTATTGCGATAGCACCATGGC-3' (SEQ ID NO: 5). Two
A2058 PCR amplified clones were subcloned into the pBL5CAT
25 (Promega) vector and sequenced to ensure the absence of PCR
artifacts. Reporter clones were then constructed in which the
only difference between the two pGL3-MMP-1 vectors was the SNP
at position -1607 bp. First, the MMP-1 promoter insert from
the pXP2 vector was subcloned into the pGL3 Basic vector
30 (Promega). Unique sequences flanking the G variation were
restricted by AatII (5') and EcoRV (3'), thereby isolating a
450/451-bp fragment from the leukocyte clone in pGL3 Basic (1
G) and A2058 melanoma DNA in pBL5CAT (2 Gs), respectively, and
excluding any other sequence variations found in the A2058
35 promoter. Finally, the 451-bp insert containing the 2 Gs was

"swapped" and ligated into the pGL3-MMP-1 construct containing
AatII/EcoRV ends, thereby generating the two SNP constructs.

Example 2: Northern Analysis

Confluent cultures in 150-mm diameter tissue culture
dishes were washed and placed in 10 ml of serum-free DMEM plus
0.2% lactalbumin hydrolysate. Immediately (time 0) and at 24
hours, total RNA was isolated using the TRIzol reagent (Life
Technologies, Inc.) and 20 µg were subjected to Northern
analysis and hybridized with cDNA-specific probes for MMP-1
or glyceraldehyde-3-phosphate dehydrogenase. Probes were
random prime labeled with $\alpha(32)$P-dCTP (12.5 µCi/reaction of
3,000 Ci/mmol) and hybridized for 20 hours at 56°C.

Example 3: Transfection and Luciferase Assay

Transient transfections were performed in triplicate in
accordance with procedures described by Rutter et al. J. Cell
Biochem. 66:322-336, 1997 with the LipofectAMINE PLUS reagent
(Life Technologies, Inc.) using 2 µg of the chimeric MMP-1
promoter/reporter plasmids, 5 µl of the PLUS reagent, and 5
µl of LipofectAMINE. Luciferase activity is reported as RLUs.
Hirt's analyses were performed and normalized to RLUs to
control for any variations in transfection efficiency.
Statistics were performed using the InStat Program (GraphPad
Software) using the Welch's alternate t test, a modification
of the unpaired t test.

Example 4: Nuclear Extract Preparation and EMSAs

Extracts of nuclear proteins were prepared, and EMSAs
were performed as described by Schroen, D.L. and Brinckerhoff,
C.E. J. Cell Physiol. 169:320-332, 1996, with 1 x 10^5 cpm of
γ^{32}P-ATP end-labeled oligo incubated with nuclear extract (5
µg) and/or recombinants ETS-1 protein (2 µM) and c-JUN protein
(1 µg; Promega). The samples were subjected to 5% PAGE at 150
V, dried, and autoradiographed. Oligos used for EMSAs were

1G sense, 5'-AAATAATTAGAAAGATATGACTTATCTCAAATCAA-3' (SEQ ID
NO: 6); 2 G sense, 5'-AAATAATTAGAAAGGATATGACTTATCTCAAATCAA:
(SEQ   ID   NO: 7)   -88/-73   sense,   5'-
TTCATTGTTAATCAAGAGGATGTTATAAAGCATGAGTCACACCCTCAGCTT-3' (SEQ
5 ID NO: 8). The -88/-73 oligo spans the region -110 to -61 bp
and includes the locations within the oligo that correspond
to the proximal PEA3/AP-1 sites at the -88/-73, respectively.

Example 5: Radiolabeled PCR Assay

Primers that flank the SNP in MMP-1 were used for PCR
10 amplification (sense primer, 5'-GTTATGCCACTTAGATGAGG-3' (SEQ
ID NO: 9); antisense primer 5'-TTCCTCCCCTTATGGATTCC-3' (SEQ
ID NO: 10)). A typical reaction consisted of ~20 ng of DNA
template; 0.2 mM dGTP, dATP, and dTTP, 2.5 µM dCTP; 10 x
buffer and Taq DNA polymerase (Sigma Chemical Co.); and
15 α(32)P-dCTP (DuPont/NEN). Reactions were PCR amplified (MJ
Research PTC100 THERMOCYCLER) in 25 cycles (4 minutes at 94°C;
45 seconds at 94°C, 45 seconds at 58°C, and 45 seconds at
72°C; followed by a brief extension (10 minutes) at 72°C).
Following amplification, the reactions (2.5 µl) were mixed
20 with 10 X loading buffer and denatured for 2 minutes at 80°C.
Samples were loaded onto an 8% denaturing PAGE and
electrophoresed for 3 hours.   Gels were dried and
autoradiographed for approximately 15 minutes.   Control
samples generated from the plasmid clones were loaded on each
25 gel for accurate scoring of the alleles. Statistics were
calculated using the InStat Program (GraphPad Software) and
were based on the percentage of the total number tested.